REMARKS

Claim Amendments:

The claims have been further amended to clarify the claims. The amendments are believed to be primarily of a clerical nature. Support for the substitution of the term "homology" with the term "identity" is found on page 3, lines 13-14. Support for new Claims 79 and 80 is found in page 2, lines 14-17, Fig. 43 and pages 18-19. Support for new Claim 81 is found in Example 35.

Objection to the Specification and Rejection of Claims 18, 20, 57-63 and 66-78 Under 35 U.S.C. § 112, First Paragraph:

The Examiner has objected to the specification and rejected Claims 18, 20, 57-63 and 66-78 under 35 U.S.C. § 112, first paragraph, on the basis of written description.

First, the Examiner contends that a biologically active fragment can be glycine that has an activity with nothing to do with ABA responses. The Examiner suggests that Applicants' may wish to amend the claims to recite "wherein said biologically active fragments participate in...". Applicants traverse this portion of the rejection and respectfully point out that the fragments recited in Claims 18, and 57-61, *as previously presented*, already recite the phrasing suggested by the Examiner. Accordingly, the recited fragments could not refer to a single glycine and clearly have the biological activity of SEQ ID NO:2. Furthermore, all of the currently pending claims recite a *specific* biological activity for the fragments. Therefore, this portion of the rejection is believed to be moot.

Second, the Examiner contends that the specification does not describe variants of SEQ ID NO:2, including those having a percent homology to SEQ ID NO:2, that *increase* ABA-mediated control of ion channels. In response, Applicants initially respectfully note that none of the previously presented claims (as presented in the July 15 Office Action) specifically recited that the protein increases ABA-mediated control of ion channels, although overexpression of the Syr protein achieves this result (see July 15 response and Example 35). The claims as previously and currently amended recite proteins that participate in ABA signalling as measured by its ability to participate in ABA-mediated control of ion channels. Applicants respectfully submit that it does not appear that the Examiner has fully considered the arguments against the written description rejection as

presented in the Amendment and Response filed on July 15, 2003. Specifically, as discussed in that response, the protein claimed by the present invention (SYR) plays a key role in mediating the signalling evoked by ABA, and it is clear from the description that the native protein and fragments or variants thereof having the biological activity of the native protein can be used to *affect or participate in*, as well as increase or augment, the ABA-mediated control of ion channels. The specification also describes a variant of Syr, which is a *truncated* syntaxin protein as claimed in Claims 78 and 79, that *inhibits* the sensitivity of the ion channels to ABA. Again, this is an antagonist variant of Syr, not the native protein. This issue is discussed again in more detail below under the discussion of the Examiner's comments regarding Claim 1. The Examiner is respectfully requested to review this section or the argument in the July 15 response for more clarification regarding the function of Syr and variants thereof.

Furthermore, Applicants traverse the Examiner's contention that the specification does not adequately describe variants of SEQ ID NO:2 as currently recited in Claims 57-60. The present specification provides a description for determining percent identity to other proteins (see page 3, line 1 to page 4, line 5), identifies proteins homologous to the SYR protein represented by SEQ ID NO:2 (see Fig. 10 and pages 40-42 of the specification), and furthermore, the specification provides detailed guidance to those of skill in the art regarding the *structural features* of a SYR protein of the invention, including *where these features are located* with regard to SEQ ID NO:2, that would readily allow one of skill in the art to recognize what changes could be made to a given SYR protein that would allow for the production of a biologically active SYR variant (e.g., see Fig. 10, Fig. 42, and pages 40-42 of the specification). Furthermore, the specification teaches that the protein with the closest identity to SEQ ID NO:2 is only 37.7% identical, and this is the Knolle gene product identified from Arabidopsis thaliana (see pages 40-42). It is clear from review of these portions of the specification, including Figs. 10 and 42, that one of skill in the art would have the guidance needed to produce sequences with up to 50% change while avoiding modifications that destroyed function. The specification also describes a SYR variant that inhibits ABA signalling (presently claimed in Claims 78 and 79 and described on pages 44-47 and in the Examples), providing additional guidance as to what changes can be made to the protein to allow this functionality. Finally, the Examples provide a detailed discussion of how one can functionally evaluate a SYR

protein or fragment or variant thereof. Therefore, it is submitted that the specification provides sufficient description to convey to one of skill in the art that Applicants were in possession of the claimed invention at the time of filing.

Finally, the Examiner contends that Claim 20 is a "reach through" claim that lacks written description. To expedite prosecution, and without any intent of prejudice to or disclaimer of the subject matter therein, Claim 20 has been cancelled.

In view of the foregoing remarks, the Examiner is respectfully requested to withdraw the rejection of Claims 18, 20, 57-63 and 66-78 under 35 U.S.C. § 112, first paragraph.

Objection to the Specification and Rejection of Claim 19 Under 35 U.S.C. § 112, First Paragraph:

The Examiner has maintained the objection to the specification and the rejection of Claim 19 under 35 U.S.C. § 112, first paragraph, contending that Claim 19 introduces new matter. The Examiner contends that the two hybrid system example, as described on pages 5-6 of the specification, describes an assay where no specific interaction between SEQ ID NO:2 and protein X is determined, but rather where a by-product reaction is determined which has nothing to do with who one would detect a specific interaction between compounds. The Examiner asserts on page 4 of the Office Action, with reference to her depiction of the two hybrid system, that "somehow all will interact with DNA to express Gal1-lacZ reporter gene."

Applicants respectfully traverse the Examiner's rejection of Claim 19 under 35 U.S.C. § 112, first paragraph.

First, outside of the reference to the two-hybrid system in the specification, the specification clearly states, on page 5, lines 29-31, that:

> "The protein of the present invention may be used in screens to detect protein-protein interactions. In particular, the protein may be used to screen for other members of a signal transduction pathway."

The specification goes on to teach, on page 6, lines 12-16, that one can detect the proteins that interact with the protein of the invention and even references an actual example of a protein that was found to interact with the Syr protein of the invention. Therefore, the specification clearly describes assays which detect protein-protein interactions, including interactions between the protein of the

invention (e.g., SEQ ID NO:2) and another member of a signal transduction pathway, such that Claim 19 does not add new matter.

Second, Applicants respectfully submit that the Examiner's reasoning in the rejection as presented in the October 21 Office Action with regard to the two-hybrid assay is fundamentally incorrect. Applicants will attempt to clarify the assay for the Examiner. As discussed in the July 15 response and in the specification, one suitable method of determining protein-protein interactions as presently claimed in Claim 19 is the two-hybrid system that is discussed on pages 5-6 of the specification. Applicants submit that the two-hybrid system is an extremely well-known system for detecting specific protein-protein interactions and is referenced in many scientific textbooks and journals (e.g., Fields and Sternglanz, *Trends Genet.* 1994 Aug; 10(8):286-292). For the Examiner's convenience, the two-hybrid system generally operates as follows: two fusion proteins are created: the protein of interest (protein X or SEQ ID NO:2 in the present case) is constructed to have a DNA binding domain attached to its N-terminus; the potential binding partner of protein X (protein Y or the signalling protein to be detected via its interaction with SEQ ID NO:2), is fused to an activation domain. If protein X interacts with protein Y (note that the requirement is an interaction between the proteins of interest, *not* their fusion partners), the binding of X to Y will form an intact and functional transcriptional activator due to the presence of the fusion partners (not the fusion of the fusion partners) and their proximity to one another *because of the binding of X to Y*. This newly formed transcriptional activator will then go on to transcribe a reporter gene, which is simply a gene whose protein product can be easily detected and measured. In this way, the amount of the reporter produced can be used as a *measure* of interaction between the protein of interest and its potential partner. Therefore, contrary to the Examiner's assertion, the two-hybrid system does measure the *specific* interaction between SEQ ID NO:2 and protein Y, and furthermore, as to the identity of protein Y, any potential binding partner can be screened using this method and indeed, one can construct a library of fusion proteins for screening using the method. Applicants are enclosing herewith a copy of an illustrated article located online at www.bioteach.ubc.ca, by Sobhanifar, Fall 2003, *BioTeach* 1(1):81, which should provide additional clarification of this assay for the Examiner (this article also references the Fields and Sternglanz citation above).

Therefore, for the Examiner to suggest that the two-hybrid system can not detect specific protein-protein interactions, and that any action recorded is "merely a by-product reaction due to the fusion of the GAL4 domains" would infer that all protein interactions that have been discovered using this method are invalid. This of course is not correct. The method of Claim 19 is fully supported by the specification, and the two-hybrid system is just one example of a technique that can be employed in the practice of the method.

In view of the foregoing discussion, Applicants respectfully request that the Examiner withdraw the rejection of Claim 19 under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 1, 2, 4-20, and 57-78 Under 35 U.S.C. § 112, Second Paragraph:

The Examiner has rejected Claims 1, 2, 4-20, and 57-78 under 35 U.S.C. § 112, second paragraph for the following reasons.

First, the Examiner contends that the acronym "ABA" renders at least the independent claims indefinite. Applicants have amended the independent claims to spell out "absicisic acid".

Second, the Examiner contends that the term "homology" is qualitative and not quantitative. Applicants have amended the relevant claims to adopt the Examiner's suggestion to substitute the term "identity" for "homology". As discussed above, the specification supports this amendment on page 3, lines 13-14.

Third, the Examiner contends that it is not clear what is the structure of the"variants" in Claim 18. Claim 18 has been amended to remove the term found objectionable by the Examiner.

Fourth, the Examiner asserts that in Claim 19, it is not clear how the interaction of the proteins is determined. Referring to the discussion of Claim 19 under the §112, first paragraph rejection, Applicants assert that methods of detecting protein-protein interactions are *well known* in the art, such that one of skill in the art, having read the claims together with the specification, would be well aware of how such an interaction can be detected. The two-hybrid system disclosed on page 5 of the description is just one example of such a method.

Finally, the Examiner contends that Claim 20 is unclear. Claim 20 has been cancelled, rendering this portion of the rejection moot.

In view of the foregoing discussion, Applicants respectfully request that the Examiner withdraw the rejection of Claims 1, 2, 4-20 and 57-78 under 35 U.S.C. § 112, second paragraph.

Rejection of Claim 20 Under 35 U.S.C. § 102(b):

The Examiner has rejected Claim 20 under 35 U.S.C. § 102(b), contending that this claim is unpatentable in view of Leung et al.

As discussed above, Claim 20 has been cancelled, without prejudice to or disclaimer of the subject matter therein and therefore, this rejection is believed to be moot.

Applicants respectfully request that the Examiner withdraw the rejection of Claim 20 under 35 U.S.C. § 102(b).

Comment on Claim 1:

Applicants note that the only rejection of Claim 1 on the record is with regard to the acronym "ABA" under 35 U.S.C. § 112, second paragraph. Therefore, Claim 1 should now be allowable. However, the Examiner indicates in the current Office Action that Claim 1 would be allowable if amended to read as set forth on page 6 of the Office Action. The only difference between the Examiner's proposed claim and Claim 1 as currently amended (and essentially as previously presented) is that the final line of Claim 1 currently reads: "wherein said protein participates in ABA signalling as measured by its ability to participate in ABA-mediated control of ion channels", whereas the Examiner suggests the following language: "wherein said protein inhibits absicisic acid (ABA) mediated control of ion channels." (emphasis added).

Applicants submit that the functional language suggested by the Examiner does not accurately represent the function of the protein of the present invention as explained in detail in the Amendment and Response filed on July 15, 2003. Since the Examiner did not respond to or specifically address this argument in the October 21 Office Action, Applicants will present the discussion again in this response. Specifically, and by way of clarification, the protein of the present invention is involved in an ABA signalling complex. As discussed on page 44, under normal conditions, the affect of ABA is to regulate ion channels (e.g., either up- or down-regulate, depending on the particular channel). The expression of the SYR protein of the present invention

(e.g., SEQ ID NO:2) has been shown by the present inventors to be regulated by ABA, and the SYR protein has been shown to participate in the control of ion channels that is mediated by ABA. For example, when the expression of the SYR protein of the invention was knocked out (see page 47-48), the response of the plant cells to ABA was lost. Similarly, when botulinum toxin was used to disable the SYR protein of the invention, ion channels showed a loss of sensitivity to regulation by ABA (see pages 44-47 and Example 27). Additionally, the effects of SYR expression in oocytes (or blocking of the same) on particular ion channel activity was demonstrated, for example, in Examples 18 and 19. Finally, overexpression of SYR in transformed plants increases sensitivity to ABA (Example 35). Therefore, the SYR protein of the present invention, including biologically active fragments and variants thereof, facilitates, allows, or even augments ABA signalling because the presence of the functional SYR protein appears to be required for normal ABA signalling.

Moreover, by identification of the SYR protein in plants, the present invention has also provided SYR proteins that *inhibit* ABA mediated control of ion channels. Such proteins would be fragments or variants of the native protein that have an inhibitory effect rather than the normal activity of the native protein (e.g., a competitive inhibitor of the native protein). The specification describes one such protein of the invention, which is a truncated syntaxin protein as presently claimed in Claims 78 and 79. When the truncated protein was used in the experiments that are summarized on pages 44-47 of the specification (and detailed in the Examples), the truncated protein inhibited the sensitivity of the ion channels to ABA.

Therefore, to make the amendment to Claim 1 as suggested by the Examiner is not correct and is therefore not acceptable to Applicants, since the function of the normal SYR protein as exemplified in the Examples is to participate in or augment ABA response, not to inhibit them. Applicants submit that Claim 1 is allowable as presently amended.

Applicants have attempted to respond to all of the Examiner's rejections as set forth in the October 21 Office Action, and it is submitted that the claims are in a condition for allowance. Applicants would like to expedite the allowance of the claims and therefore, if the Examiner has any additional concerns regarding the claims or would like to have any additional clarification of the

arguments presented herein, the Examiner is encouraged to contact the below-named agent at (303) 863-9700 to discuss the same.

Respectfully submitted,

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Date: January 22, 2004

The Yeast Two-Hybrid Assay
An Exercise in Experimental Eloquence

Solmaz Sobhanifar
Graphics: Jiang Long

Once upon a time, it was believed that proteins were isolated entities, floating in the cytosol and, for the most part, acting independently of surrounding proteins. Proteins were thought to diffuse freely, and reactions occurred as a result of proteins A and B randomly colliding with one another. Today we know this picture to be far too simplistic to account for the complex processes that all coalesce to be 'life'. Instead, the majority of cellular phenomena are carried out by protein 'machines', or aggregates of ten or more proteins[1]. These protein-protein interactions are critical to all cellular processes, and understanding them is key to understanding any biological system. One technique that can be used to study protein-protein interactions is the "yeast two hybrid" system.

Yeasty goodness

A protein is composed of modules or domains, which are individually folded units within the same polypeptide (protein) chain. The presence of these individual domains allow the same protein to perform different functions. The yeast two-hybrid technique uses two protein domains that have specific functions: a DNA-binding domain (BD), that is capable of binding to DNA, and an activation domain (AD), that is capable of activating transcription of the DNA.

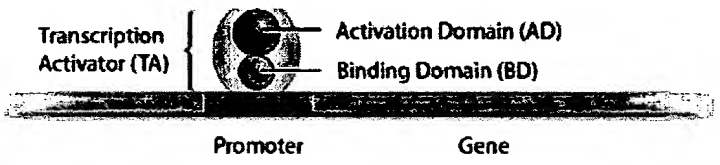


Figure 1. Normal Transcription. Normal transcription requires both the DNA-binding domain (BD) and the activation domain (AD) of a transcriptional activator (TA).

Both of these domains are required for transcription, whereby the DNA is copied in the form of mRNA, which is later translated into protein. In order for DNA to be transcribed, it requires a protein called a transcriptional activator (TA). This protein binds to the "promoter", a region situated upstream from the gene (coding region of the DNA) that serves as a docking site for the transcriptional machinery (Figure 1). Once the TA has bound to the promoter, it is then able to activate transcription via its activation domain. Hence, the activity of a TA requires both a DNA binding domain and an activation domain. If either of these domains is absent, then transcription of the gene will fail.

Furthermore, the binding domain and the activation domain do not necessarily have to be on the same protein. In fact, a protein with a DNA binding domain can activate transcription when simply bound to another protein containing an activation domain; this principle forms the basis for the yeast two-hybrid technique[2].

In the two-hybrid assay, two fusion proteins are created: the protein of interest (X), which is constructed to have a DNA binding domain attached to its N-t rminus, and its potential binding partner (Y), which is fused to an activation domain. If protein X interacts with protein Y, the binding of these two will form an intact and functional transcriptional activator[2]. This n wly formed transcriptional activator will then go on to transcribe a reporter gen , which is simply a g ne whose protein product can be easily detected and measured. In this way, the amount of the reporter produced can be used as a measure of interaction between our protein of interest and its potential partner (Figure 2).

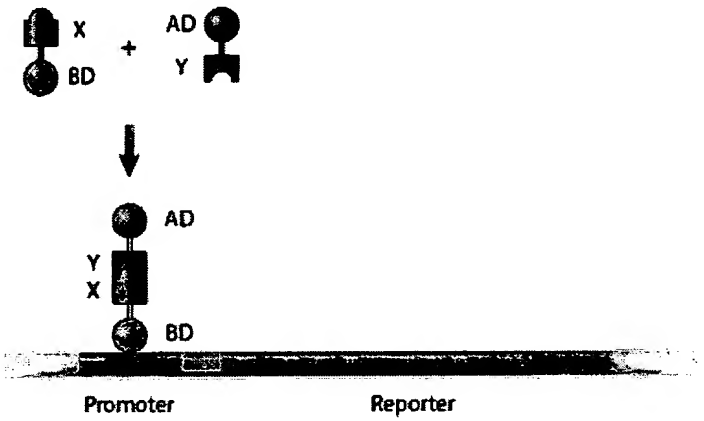
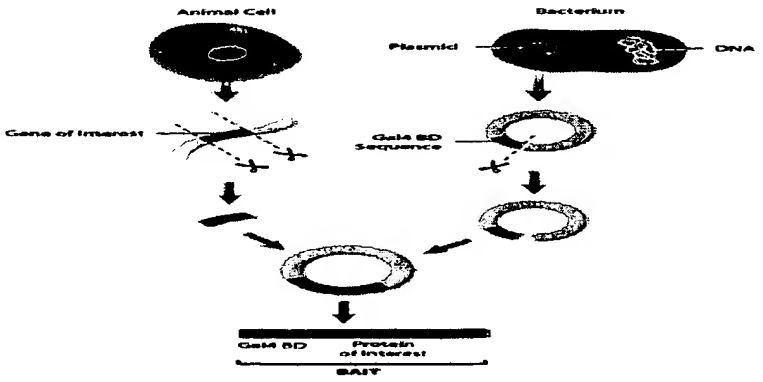


Figure 2. Yeast two-hybrid transcription. The yeast two-hybrid technique measures protein-protein interactions by measuring transcription of a reporter gene. If protein X and protein Y interact, then their DNA-binding domain and activation domain will combine to form a functional transcriptional activator (TA). The TA will then proceed to transcribe the reporter gene that is paired with its promoter.

The Recipe for Interactions

First, it is necessary to construct the 'bait' and 'hunter' fusion proteins. The 'bait' fusion protein is the protein of interest (or 'bait') linked to the GAL4 BD. This is done by inserting the segment of DNA encoding the bait into a plasmid, which is a small circular molecule of double-stranded DNA that occurs naturally in both bacteria and yeast. This plasmid will also have inserted in it a segment of Gal4 BD DNA next to the site of bait DNA insertion. Therefore, when the DNA from the plasmid is transcribed and converted to protein, the bait will now have a BD attached to its end (Figure 3). The same procedure is used to construct the 'hunter' protein, where the potential binding partner is fused to the GAL4 AD.



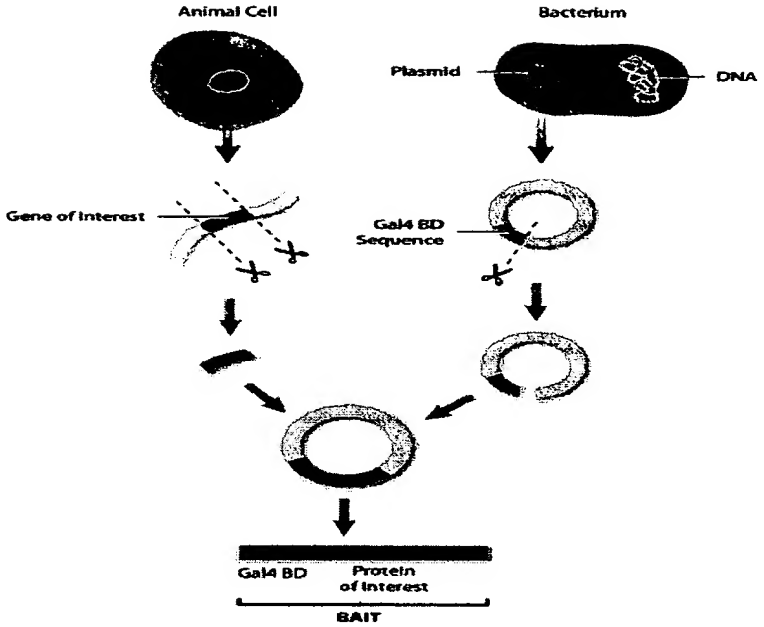


Figure 3. Plasmid construction. The 'bait' and 'hunter' fusion proteins are constructed in the same manner. The 'bait' DNA is isolated and inserted into a plasmid adjacent to the GAL4 BD DNA. When this DNA is transcribed, the 'bait' protein will now contain the GAL4 DNA-binding domain as well. The 'hunter' fusion protein contains the GAL4 AD.

In addition to having the fusion proteins encoded for, these plasmids will also contain selection genes, or genes encoding proteins that contribute to a cell's survival in a particular environment. An example of a selection gene is one encoding antibiotic resistance; when antibiotics are introduced, only cells with the antibiotic resistance gene will survive. Yeast two-hybrid assays typically use selection genes encoding for amino acids, such as histidine, leucine and tryptophan (Figure 4).

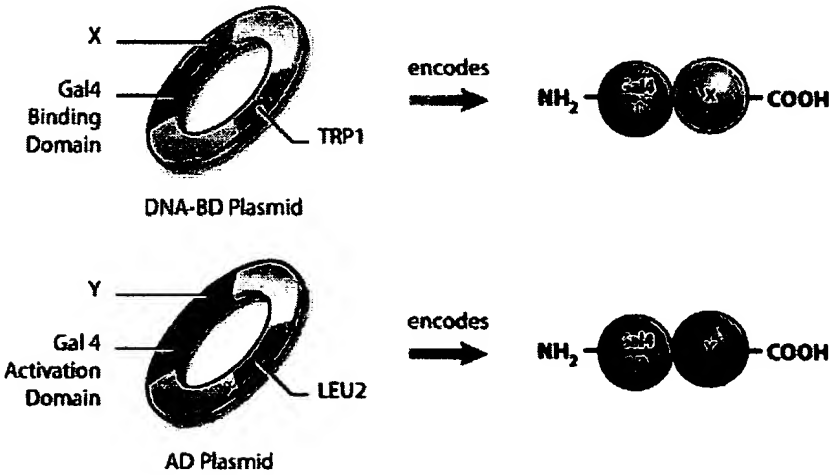


Figure 4. Title. The yeast two-hybrid assay uses two plasmid constructs: the bait plasmid, which is the prot in of interest fused to a GAL4 binding domain, and th hunter plasmid, which is the potential binding partner fused to a GAL4 activation domain.

Once the plasmids have been construct d, they must next be introduced into a host yeast cell by a process called "transf ction". In this process, the outer-membrane of a yeast cell is disturbed by a physical method, such as sonification or chemical disruption. This disruption produces holes that are large enough for the plasmid to enter, and in this way, the plasmids can cross the membrane and enter the cell (Figure 5).

Once the cells have been transfected, it is necessary to isolate colonies that have both 'bait' and 'hunter' plasmids. This is because not every cell will have both plasmids cross their plasma membrane; some will have only one plasmid, while others will have none. Isolation of transfected cells involves identifying cells containing plasmids by virtue of their expressing the selection genes mentioned previously. After the cells have been transfected and allowed to recover for several days, they are then plated on minimal media, or media that is lacking one essential nutrient, such as tryptophan. The cells used for transfection are called auxotrophic mutants; these cells are deficient in producing nutrients required for their growth. By supplying the gene for the deficient nutrient in the 'bait' or 'hunter' plasmid, cells containing the plasmid are able to survive on the minimal media, whereas untransfected cells cannot (Figure 5). Selection in this way occurs in two rounds: first on one minimal media plate, to select for the 'bait' plasmid, and then on another minimal media plate, to select for the 'hunter'[4].

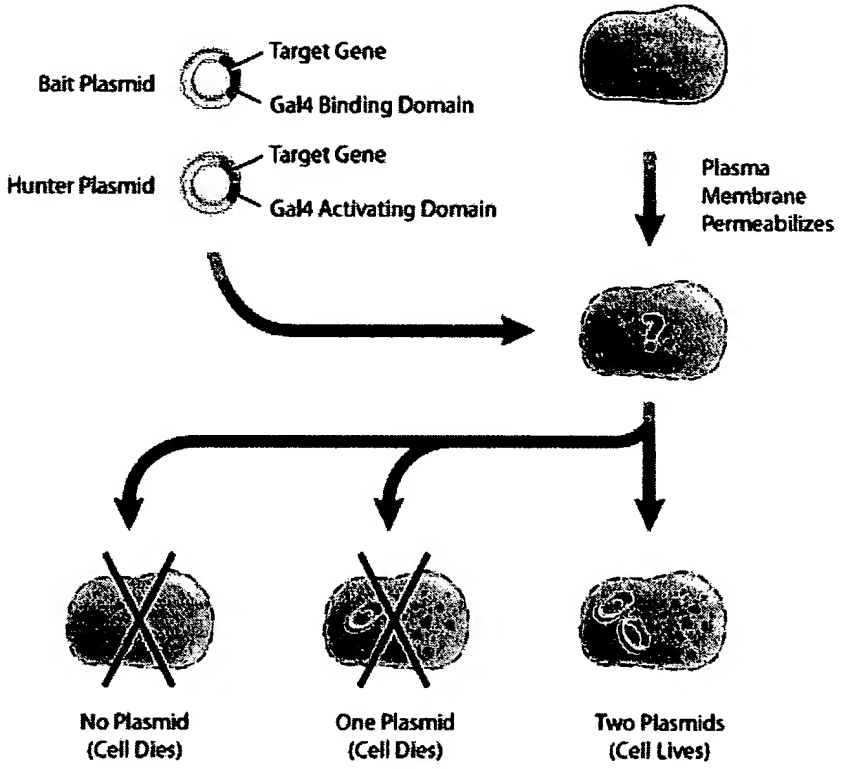


Figure 5. Transfection. The 'bait' and 'hunter' plasmids are introduced into yeast cells by transfection. In this process, the plasma membrane is disrupted to yield holes, through which the plasmids can enter. Once transfection has occurred, cells containing both plasmids are selected for by growing cells on minimal media. Only cells containing both plasmids have both genes encoding for missing nutrients, and consequently, are the only cells that will survive.

Once inside the cell, if binding occurs between the hunter and the bait, transcriptional activity will be restored and will produce normal Gal4 activity. The reporter gene most commonly used in the Gal4 system is LacZ, an *E. coli* gene whose transcription causes cells to turn blue[4]. In this yeast system, the LacZ gene is inserted in the yeast DNA immediately after the Gal4 promoter, so that if binding occurs, LacZ is produced. Therefore, detecting interactions between bait and hunter simply requires identifying blue versus non-blue.

What Can I Do With My V ry Own Y ast Two-Hybrid??

Firstly, the yeast two-hybrid assay can identify novel protein-protein interactions. By using a number of different proteins as potential binding partners, it is possible to d tect interactions that were pr viously uncharacterized[3]. Secondly, the yeast two-hybrid assay can be used to characterize interactions already known to occur. Characterization could include determining which protein domains are responsible for the interaction, by using truncated proteins, or under what conditions interactions take place, by altering the intracellular environment.

The last and most recent application of the yeast two-hybrid involves manipulating protein-protein interactions in an attempt to understand its biological relevance. For example, many disorders arise due to mutations causing the protein to be non-functional, or have altered function. Such is the case of some cancers; a mutation in a pro-growth pathway does not allow for the binding of negative regulatory proteins, resulting in the pro-growth pathway never turning 'off'. The yeast two-hybrid is one means of determining how mutation affects a protein's interaction with other proteins. When a mutation is identified that affects binding, the significance of this mutation can be studied further by creating an organism that has this mutation and characterizing its phenotype.

Conclusion...

The yeast two-hybrid assay is an eloquent means of investigating protein-protein interactions. A fairly new addition to the family of biological studies, these interactions have become increasingly important to our understanding of biological systems in the past few years. While isolation of a protein in an attempt to understand its function still has its place in biological research, we now understand that biological reactions do not occur in isolation. A protein is constantly interacting with other proteins in what we now know to be a delicate balance — to ignore this wealth of information would be to deny ourselves the opportunity to fully appreciate the stuff that life is made of.

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Additional Reading

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